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STUDIES ON PREMATURELY SENILE RATS

DISSERTATION

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It is with much pleasure that the writer takes this opportunity to express her appreciation and gratitude for the inspiration and help given to her by Dr. E. V. McCollum, and also to Dr. William H. Howell for his many kindnesses.

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Part 1

INTRODUCTION

The Department of Chemical Hygiene has for some years been developing a comprehensive plan of investigation, one of the objects of which is to perfect methods for the evaluation of each of the essential dietary factors which is concerned with the nutrition of mammals. At present quality and quantity of protein are recognized as important factors in determining the value of a diet. Other essential factors which are of practical importance are: a proper adjustment of the several mineral elements which the diet must furnish, and the three unidentified food factors which are respectively responsible for the prevention of xerophthalmia, polyneuritis, and scurvy. It cannot be said that any one of these factors is of greater importance than another, except that failure will supervene somewhat sooner in one case than in another, although this depends almost entirely on the extent of the deficiency.

As a phase of this general plan, a series of experiments was carried out in 1919-1920 with the view to comparing with as great accuracy as possible the biological values of a series of proteins from different sources. In carrying out this study, certain principles not hitherto employed were made use of in order to heighten the sensitiveness of the test. It was learned in earlier studies that if the quality of the protein is excellent, young rats are able to grow in a normal manner when the food mixture contains but nine per cent of this factor. The animals also show high fertility and fairly good

success in the rearing of young. They are also able to remain vigorous to an age of approximately twenty to twenty-four months before exhibiting marked signs of deterioration.

If the quality of the protein in the diet is not of the best, and but nine per cent is included in the food mixture, the growth may be normal, but the animals show lowered capacity in all the other functions enumerated above. The extent to which they fail in fertility, or show high infant mortality, or age early, forms a very sensitive method of comparing the values of proteins from different sources when these are fed at this plane of intake, supplemented in a fairly satisfactory manner with respect to all other dietary factors.

In order to facilitate the comparison of the various diets, the value of the protein mixture when fed at a constant level of nine per cent of the total food intake, has been judged according to an arbitrary scale. Protein mixtures which support an animal in the manner described in the preceding paragraph were called "excellent"; those which produce normal growth, a high fertility, high infant mortality, and a moderately early aging (old at about 16 months) were marked "good"; those which produce undersized animals (old at 12 or 14 months), and having a low fertility and high infant mortality, were marked "poor". There are, of course, all gradations between these three classes, but in general the protein mixtures fall into these groups. Chart showing the life cycle of typical animals fed on proteins of these 3 types is appended.

These criteria afford a means of determining approximately how old the animals on a series of diets are in respect to the vigor

and normality of their tissues, as well as their physiological age. We have still other possibilities for determining the actual physiological condition of experimental animals. Thus we may reasonably expect an aged animal, irrespective of whether it has lived long and has developed the signs of age slowly, or has lived but a short time and aged rapidly, to show certain changes in its tissues which are concomitant with deterioration of the body structures. Folin (1) has described data which seem to indicate that the efficiency of the kidneys tends to decrease progressively from youth to old age, and as a result the end-products of protein metabolism tend to remain in the blood and lymph in greater amounts as age advances. Analysis of the blood for these products, which are usually designated collectively as "non-protein nitrogen", should be of value in connection with other criteria as to the actual physiological age of the animal. A certain amount of anemia may be expected in animals which have become debilitated to a serious extent. In fact, any analytical data of high accuracy on animals which are in a poor physical condition is of distinct value when it can be compared with similar data on youthful and normal animals.

It was also thought that interesting results might be obtained by a study of the calcium in the blood, as it had been observed that many of the animals on these diets had brittle teeth and bones.

The present paper deals with the chemical analysis of the blood and the data collected at autopsy, on animals representing

groups in a series in which in most cases the sole fault in the diets was in the protein moiety, although in a few of the lots there was a deficiency of calcium or fat-soluble A, or both. A list of the diets is given in the tables in the appendix.

It was hoped that the data collected on the animals on diets containing nine per cent protein might contribute something to the controversy over the value of low and high protein diets which has been debated for so many years. Formerly the data was obtained either by a large number of dietary studies or by nitrogen balance experiments. No account was taken of the quality of the protein or of the prolonged well-being of the subject. The early workers in this field, Voit, Rubner, and Atwater (2) from evidence obtained by the statistical method, recommended 118 gms. to 127 gms. (about 15 per cent of a 3000 calorie diet) as the proper allowance for men doing active work. This tradition was questioned about 1904 by Chittenden and his co-workers (3), who advised an intake of 36 gms. to 40 gms. of protein (about 7 per cent of the total diet) per day for active men. These figures were based on experiments lasting five months, during which time the men were in good health and maintained their nitrogen balance. A similar diet is recommended by Hindhede (4). On the other hand McCay (5), after a study of the diet and physical and mental condition of the races of India, concludes that "the higher the level of protein interchange the more robust and energetic and the more manly the race."

The most recent work on a large scale is that carried on by Benedict and his co-workers (6) during 1918 and 1919 on a squad of young men. A typical diet contained about 55 gms. of protein and 1600 calories. Benedict stated that there are "definite and significant disturbances of blood composition, normal sex expression and neuro-muscular efficiency and ..... mental and physical unrest", and yet the conclusion was drawn by the writers that "protein curtailment is an assured and physiologically sound procedure." Apparently the ill effects of the diet are blamed entirely on the low calorie content, although there is no proof that it is due to this or some other factor in the diet such as the low protein content, or to both. The duration of the experiment, like that of Chittenden, is but for a very small fraction of the span of life, and so the evidence of the efficiency of their diets in promoting normal nutrition cannot be taken as conclusive. Moreover, the careful measurements of Benedict indicate that some conditions, at least, were met which were similar to those found on animals kept on diets containing 9 per cent protein.

Part II

PROCEDURE AND METHODS.

The rats for this study, 142 in all, were selected when they were between the ages of 200 and 600 days. The animals were

divided into 45 lots, each lot representing a different diet. In 13 lots (containing 37 rats) the animals were on maintenance diets containing various amounts of protein. Some were low in calcium and fat-soluble A. (For a complete record of the diets see the tables in the appendix). In all the other lots the protein was the only deficiency. It was always present in an amount equal to 9 per cent, and in quality complied with one of the three grades mentioned in the introduction.

The animals on the diets producing very early aging, and those representing the second or third generations on defective diets, were killed when they were between the ages of 200 and 350 days, because they would not have survived much longer. One animal was 711 days old. None had reached the end of the normal span of a rat's life (about 900 days), and even on the best of these diets the animals were usually in a poor condition before they reached 600 days.

The rats were given no food during 8 hours prior to being killed. They were weighed, etherized, and samples of blood taken from the end of the tail for erythrocyte and leucocyte counts and hemoglobin determinations. An incision was then made and blood drawn directly from the heart with a syringe or suction apparatus made by attaching a hyperdermic needle to the end of a 10 cc. graduated pipette. Since calcium was determined in most of the samples, sodium citrate was used to prevent clotting. A few

pulverized crystals were put into the syringe or drawn up into the pipette.

Observations were made on the general condition of the animal, and designated as "good", "fair", or "poor", on the appearance of the hair and skin and the brittleness of the teeth. The amount of superficial and body fat, and the condition of the lungs and other organs, was also noted. The lungs were marked normal +, ++, +++, ++++, according to the presence and extent of the lung infection from which many of them suffered. The heart, liver, kidneys and spleen were removed, and each was weighed.

Blood cell counts were made in the usual manner. The per cent hemoglobin was measured with a Sahli hemometer having a scale such that an individual having a hemoglobin content of 14.3 gms. per 100 cc. of blood gave a reading of 87 per cent. Corrections were accordingly made on each reading so that 14.3 gms. of hemoglobin per 100 cc. of blood corresponded to 100 per cent.

Chemical Examination of the Blood. To precipitate the blood proteins a 10 per cent trichloracetic acid solution was used according to the methods of Folin and Wu, and others (7, 8, 9). One part of blood was precipitated by nine parts of trichloracetic acid. The precipitates were allowed to stand for at least 6 hours, then filtered and preserved with two drops of toluene (7). Non-protein N was determined according to the method of Folin and Wu (7). Practically all the determinations were made in duplicate.

For the standard $(NH_4)_2SO_4$ was decomposed with concentrated NaOH and the NH_3 aspirated through H_2SO_4. The $(NH_4)_2SO_4$ was precipitated with alcohol, filtered, and the crystals dried. They were purified by dissolving and reprecipitating twice.

Calcium was determined in the trichloracetic acid filtrate, a combination of the method by Lyman (10) and Halverson and Bergeim (11), (12) being used. Lyman recommends the use of 6.5 per cent trichloracetic acid as a precipitant, but equally accurate results were obtained with the 10 per cent solution (Table 1). An amount of filtrate containing approximately 2 cc. of blood was measured into a 50 cc. flask and made alkaline with 2 N NH_4OH, using methyl orange as an indicator. The solution was neutralized with $\frac{5}{100}$ N HNO_3 made acid with 1 cc. of 4 per cent oxalic acid and then 1 cc. of 20 per cent sodium acetate was added. The flask was stoppered, the contents cooled under the hydrant, shaken until a faint precipitate appeared, and kept in a refrigerator over night. The contents of the flask were centrifuged in a tube which had been drawn out to a point. The clear, supernatant liquid was drawn off with a capillary pipette. The precipitate was washed with 5 cc. of distilled H_2O, recentrifuged, and the H_2O drawn off. This was rep ated three times. The precipitate was then dissolved in 5 cc. of 2 per cent H_2SO_4, diluted to 25 cc. and titrated with $\frac{n}{100}$ $KMnO_4$. This method gave excellent results with known small quantities of calcium. The trichloracetic acid did not interfere as is shown by Table I.

ACCURACY OF THE METHOD FOR DETERMINING CALCIUM

	Mgs. calcium recovered	Mgs. calcium theoretical
30 cc. standard calcium solution	0.59	0.6
20 cc. ditto.	0.41	0.4
20 cc. "	0.4	0.4
20 cc. "	0.4	0.4
15 cc. "	0.29	0.3
10 cc. "	0.2	0.2
20 cc. standardcalcium solution + 25 cc 10% trichloracetic acid	0.4	0.4
20 cc. ditto.	0.42	0.4
20 cc. "	0.42	0.4
10 cc. "	0.2	0.2

Table 1.

Part III

DISCUSSION OF RESULTS

Autopsy Findings

The coat of a normal rat is sleek and well kept, the hairs are of even length, the skin is clean, and there is a considerable amount of body fat. None of the experimental animals could be considered in excellent condition, and only four were in a good condition. The latter were from lots 2188, 2578, 2180 and 1895 respectively, but since other animals on these same diets were in a poor condition these four cases out of the 142 animals examined cannot be considered typical, but represent, rather, exceptions to the rule. It is evident that at the level fed the proteins of these diets are conducive to a loss of body weight and a poor general condition during the prime of the animal's life. This loss of weight is due not only to a decrease in fatty tissue, but also to a decrease in muscular and organ tissue. The muscles of the legs become stringy. Those between the ribs are often like a very thin sheet. The weights of the liver, kidneys, spleen and heart respectively vary greatly in different animals, but in general they are roughly in proportion to the body weight, those in poor condition having a low body weight and small organs. (The weights of all the animals and of the organs are given in the tables in the appendix).

The Genital Organs. Special mention should be made of the condition of the genital organs because of the low fertility of many of the animals. In many of the males the testes were markedly

undersized, and many of the females had a purulent or hydra salpingitis, and others, a cystic degeneration of the ovaries.

Of the 66 males on which autopsies were performed, 29 (44 per cent) had small testes or seminal vesicles, and in many cases both had degenerated. Of the 76 females, 24 (31.5 per cent) had infections of the ovaries or uteri, or both, and 4 animals had undersized but uninfected ovaries and uteri. Since no histological studies were made it is not possible to determine whether the low fertility was due to a lack of active spermatozoa or to the infections in the female. It is not unlikely that both factors enter in. In those lots showing a high fertility, only 1 of the 13 males had small testes, and only 2 of the 11 females had an infection. In those lots showing a low fertility 19 of the 36 males (53 per cent) had small genitals and 13 of the 42 females had infections, and 2 had very small ovaries.

Little work has been done on the relation of diet to sterility in rats. Osborne and Mendel (13) report that with a few exceptions animals were sterile when kept on diets in which 30 per cent and 40 per cent of yeast respectively supplied the protein. The diet was properly supplemented in all other respects. The growth curve was normal. They reported autopsies on only 2 males, and these had testes of from one-quarter to one-third the normal size. As in our animals, the protein content was the only doubtful factor in the diet. Drummond (14) and Funk and Douglas (15) have reported that sterility occurs on a diet deficient in water-soluble B. Only one

lot (2286) consisting of 2 males and 3 females, were on a diet low in this factor, and in this lot 1 male had only one testis, the other male appearing normal, while the fertility of the females was low.

Lung Infections. It has long been known that rats are particularly susceptible to lung infection. Hektoen (16) has described 2 types of lesions, one being solid and red, usually with nodular reddish-yellow or yellow areas, and riddled with abscesses containing a thick yellowish pus. In the second type, the lesions had sharp but irregular margins containing a gelatinous, translucent grey, or opaque yellow fluid. The gelatinous appearance corresponds to the appearance of the exudate in broncho-pneumonia. Two types of lesions were observed in our animals, the one having nodular areas filled with a hard yellow or greenish-yellow cheesy mass, the second being similar to the second type observed by Hektoen. No reddish nodules were observed, although occasionally a lung otherwise normal appeared hemorrhagic. Almost all the lungs had authracosis. Of the total number of rats examined, 36 (25 per cent) had normal lungs. Apparently the condition of the animal was not the only predisposing factor because 10 of the 36 animals were in fair condition, and 2 were in good condition. There were only 5 of the 45 lots in which all the rats had normal lungs, and of the five, three contained only one rat each, so no conclusions can be drawn from them.

STUDIES ON THE BLOOD

Calcium. An abnormal calcium metabolism is frequently cited

as one of the concomitants of old age. The so-called hardening of the arteries due to the deposition of calcium is a very common occurrence in the aged. McCallum in his Textbook of Pathology (17) speaks of a profound difference between the calcium metabolism of youth and of old age. He states that more calcium is absorbed from the bones than is deposited there afresh - a condition of senile osteoporosis. In cases of osteomalacia the histological picture is the same as in rickets, except that the endochondrial ossification is complete. McCallum says that the cause is debated, but that it is thought to be due to an adrenal insufficiency or, in women, to an ovarian hyperactivity. There is no suggestion that the cause or one of the causes may be a dietary deficiency, as in the case of rickets.

It was stated in the introduction on page 3 that many of the animals showing signs of premature old age had poor teeth. Some pulled out easily from the gums. Others were so brittle that if grasped between the forceps they would break in two. This was true of almost half (44 per cent) of the animals. In some cases (30 per cent) the ribs were brittle, in others they were as flexible as cartilage. So it seemed probable that there had been some derangement of the calcium metabolism. The occurrence of high calcium content in the blood and the brittleness of teeth and ribs is shown in Table II.

The normal calcium content of the rats' blood was found to be about the same as for human beings - between 7.4 mgs. and 11.1 mgs. calcium per 100 cc. blood. Halverson and his co-workers (18) give

Table II

Lot No.	Percentage protein	Character of protein	Calcium in the blood mgs./100 cc	Condition of teeth	Condition of ribs
2195	9	good	20.5	brittle	very flexible
2195	9	"	----	"	brittle
2191	9	poor	15.6	O. K.	O. K.
2191	9	"	----	brittle	brittle
2190	9	"		O. K.	very flexible
2188	9	good	13.1	sl. brittle	very flexible
2188	9	good	----	brittle	brittle
2195	9	poor	11.2	"	O. K.
2195	9	"	10.9	O. K.	O. K.
2180	9	"	14.5	brittle	O. K.
2180	9	"	16.8	"	O. K.
2180	9	"	14.0	"	O. K.
2179	9	good	10.1	O. K.	O. K.
2179	9	"	8.8	O. K.	O. K.
2179	9	"	----	brittle	O. K.
2177	9	poor	22.	"	brittle
2178	9	"	----	"	"
2379	9	"	9.5	O. K.	brittle
2379	9	"	10.8	brittle	O.K.
2379	9	"	9.0	O.K.	O.K.
2378	9	good	15.6	O. K.	brittle
2378	9	"	26.1	O. K.	"
2371	9	poor	9.8	O. K.	O. K.
2371	9	"	10.2	O. K.	O. K.
2364	9	"	13.3	brittle	O. K.
2369	9	"	11.1	"	brittle
2363	9	"	----	"	O. K.
2346	9	good	20	"	O. K.
2362	9	excellent	14.0	"	O. K.
2362	9	"	15.0	"	O. K.
2370	9	"	8.8	loose at gums	brittle
2370	9	"	----	brittle	"
2390	9	poor	----	"	"
2388	9	"	8.1	O. K.	brittle
2388	9	"	----	brittle	"

Table II (continued)

Lot No.	Percentage protein	Character of protein	Calcium in the blood mgs./100 cc	Condition of teeth	Condition of ribs
2388	9	poor	----	brittle	brittle
2387	9	good	9.8	O. K.	"
2387	9	"	9.6	brittle	" *
2389	9	poor	9.5	O. K.	"
2389	9	"	8.0	O. K.	"
2181	9	good	9.8	O. K.	O. K.
2185	9	poor	----	O. K.	O. K.
2491	9	"	7.6	O. K.	brittle
2491	9	"	10.5	brittle	"
2491	9	"	7.5	loose at gums	"
2494	9	"	----	brittle	"
2474	9	"	6.1	"	O. K.

* enlarged costochondrial junction.

a content of between 9 mgs. and 11 mgs. per 100 cc. blood for normal men and women (30 cases) and Jansen (19) reports a content of 11.5 mgs. to 12 mgs. per 100 cc. of blood in middle-aged individuals. Determinations were made on the blood of 57 of the rats studied, and of this number 25 were above 11 mgs. and 2 were as high as 22 mgs.

The relation between the calcium content of the blood, the brittleness of the teeth, and the brittleness or extreme flexibility of the ribs is not altogether clear. However, no animal with a high calcium content in the blood had normal teeth and ribs, although some animals with brittle teeth and ribs had a normal amount of calcium in the blood.

It seems probable that this abnormal calcium metabolism is linked with the protein content of the diet. With one exception there was no lot (of those in which the diets contained 9 per cent protein) in which all the animals had a normal calcium content in the blood and normal teeth and ribs. (In the exception there was only one animal in the lot, so it was not significant). These abnormalities could not be due to a lack of calcium in the diet, as the calcium of the foodstuffs was supplemented by the addition of 1/5% $CaCO_3$, making the total calcium content of the diets between .6 per cent and .75 per cent, an amount sufficient to meet the needs of the animals. It is evident, therefore that 9 per cent of even the best combination of proteins from the standpoint of growth is not sufficient to enable the body to carry on a normal calcium metabolism throughout life. McCollum and Park and their co-workers (verbal communication) in their work on

the etiology of rickets have produced defective bone formation on diets having sufficient calcium, but containing inadequate protein. There is no evidence in this study that the inadequacy of the protein directly affects the bony structures or if it acts by producing changes in the endocrine organs as suggested by MacCallum.

Seven animals were studied which had been living on diets containing 22 per cent of protein but low in fat-soluble A, and with and without an adequate amount of calcium. Table III shows the effect of such a diet on the calcium content of the blood and the condition of the teeth and ribs.

Although the number of animals studied in this connection is small, the results suggest probability that besides the protein, fat-soluble A is also concerned in calcium metabolism. This also is in agreement with the work of McCollum and his collaborators on rickets, in which they show that a deficiency of fat-soluble A in the diet causes faulty bone formation (verbal communication). That the fat-soluble A, as well as calcium, is concerned is shown by the animals in lot 2405 having a diet in which a deficiency of this vitamine is the only fault. These animals have an abnormally high calcium content in the blood, and both the teeth and ribs are abnormal in texture. A paper by Wells and Benson (20) is very suggestive in this connection. They studied the ossification of foetal epiphyseal cartilage by implanting it in the peritoneal cavity of rats, and they concluded from this work that the ability of these cells to take up calcium depends on the presence of some non-living constituent.

Lot No.	Deficiency	Ca. in blood mgs. per 100 cc.	Teeth	Ribs	Notes
2750	low Ca. and low fat-sol. A.	18.7	brittle	brittle	
2750	low Ca. and low fat-sol. A.	10.6	normal	brittle	enlarged costochonurial junction
2406	low Ca. and low fat-sol. A.	21.5	brittle	brittle	rickets in 2nd and 3rd generations.
2406	low Ca. and low fat-sol. A.	12.2	brittle	brittle	rickets in 2nd and 3rd generations.
2405	low fat-soluble A.	18.6	brittle	flexible	
2405	low fat-soluble A.	19.0	brittle	flexible	
2405	low fat-soluble A.	16.5	brittle	flexible	

Table III

The Erythrocyte Count and Hemoglobin Content. The erythrocyte count and the percentage of hemoglobin serves as another index of the condition of these animals. The red count of normal stock rats from this laboratory varies between 8,000,000 and 10,000,000, although occasionally an animal will be found with a count as high as 12,000,000. The hemoglobin content may be as high as 144 per cent in vigorous young males weighing about 350 grams. The average is about 130 per cent. Donaldson (21) quoting Rivas gives the erythrocyte count of the normal albino as between 7,400,000 and 9,200,000, and the amount of hemoglobin between 85 per cent and 100 per cent. It should be noted, however, that these animals had been fed on garbage (personal communication) and were of inferior stock. Five vigorous males of this same stock reared on a diet known to be adequate gave hemoglobin readings between 123 per cent and 137 per cent.

Of the 123 erythocyte counts made, 74 (61 per cent) were above 7,500,000. Some of those below this figure were very low; two animals, for example, having a count of 2,500,000 (Table IV).

It will be seen from the table that most of the animals on the 9 per cent protein diets have high counts. The exceptions are lots 2379, 2371, 2390, 2388, 2491 and 2494, in all of which a legume seed is supplemented with either a cereal, dried skim milk or steak. Some of the animals, however, on diets containing a legume have normal counts. The lowest counts are those made on the animals on maintenance diets, all the very low counts being from these groups. Of the 15 animals on maintenance diets only 2 had counts above 8,000,000, the average of those

Table IV.

Lot No.	Percentage protein	Character of protein	Erythrocytes millions per cmm.	Hemoglobin percentage	Notes
2195	9	good	11	121	
2195	9	"	12	132	
2195	9	"	9.5	109	
2191	9	poor	9	100	
2191	9	"	9	97	
2191	9	"	7	107	
2190	9	"	7	104	
2190	9	"	8	104	
2190	9	"	9	109	
2190	9	"	9	115	
2190	9	"	9.5	121	
2184	9	"	8	98	
2184	9	"	8	87	
2184	9	"	7.5	98	
2188	9	good	9	101	
2188	9	"	7	104	
2183	9	poor	8	108	
2195	9	"	9.5	112	
2195	9	"	8.5	107	
2195	9	"	9	102	
2195	9	"	9	110	
2180	9	"	9	107	
2180	9	"	9.5	117	
2180	9	"	12	120	
2179	9	good	8	109	
2179	9	"	11	99	
2177	9	poor	7.5	104	
2178	9	"	8.0	109	
2379	9	"	7.5	99	
2379	9	"	7.5	91	
2379	9	"	5.5	100	
2378	9	good	8	106	
2378	9	"	8	121	
2371	9	poor	6	98	
2371	9	"	6.5	98	
2364	9	"	9	115	

Table IV. (continued)

Lot No.	Percentage protein	Character of protein	Erythrocytes millions per cmm.	Hemoglobin percentage	Notes
2364	9	poor	10.5	115	
2364	9	"	10	113	
2364	9	"	9	137	
2364	9	"	9	118	
2364	9	"	10	124	
2364	9	"	8	121	
2369	9	"	8.5	106	
2363	9	"	9.0	121	
2363	9	"	8	92	
2363	9	"	8	113	
2363	9	"	9	121	
2346	9	good	7	104	
2362	9	excellent	8	92	
2570	9	"	12	---	
2370	9	"	10	---	
2370	9	"	8.5	---	
2370	9	"	4.5	---	
2390	9	poor	6	69	
2390	9	"	7	86	
2390	9	"	8/5	113	
2388	9	"	8	92	
2388	9	"	8.5	99	
2388	9	"	5.5	83	
2388	9	"	9	92	
2387	9	good	7	109	
2387	9	"	7	104	
2387	9	"	8	115	
2387	9	"	9	121	
2387	9	"	--	92	
2389	9	poor	7	98	
2181	9	good	8.5	100	
2185	9	poor	8	94	
2491	9	"	4	90	
2491	9	"	7.5	98	
2491	9	"	6.5	92	
2491	9	"	6.0	86	
2494	9	"	5	74	
2484	9	"	7.5	98	
2474	9	"	8	92	
1894	8	"	6	69	

Table IV. (continued).

Lot No.	Percentage protein	Character of protein	Erythrocytes millions per cmm.	Hemoglobin percentage	Notes
1895	9	poor	8	93	
1896	10	"	8	69	
1896	10	"	6.5	109	
1896	10	"	4.0	29	
2437	16	"	8	115	
2437	16	"	3.5	---	
2437	16	"	7	109	
2437	16	"	3.5	---	
2437	16	"	6	121	
2437	16	"	8	---	
2340	12	"	3.5	86	
2340	12	"	3.5	69	
2340	12	"	6.0	104	
2343	18	good	7	104	
2343	18	"	7	---	
2343	18	"	6	89	
2414	35	"	6.5	101	
2417	20	fair	6	90	
2750	22	good	2.5	63	low Ca, low fat-soluble A.
2750	22	"	1.4	26	
2750	22	"	3.2	52	
2127	7 1/2	poor	10	95	
2127	7 1/2	"	8	90	
2175	7	"	7.5	118	
2175	7	"	6	94	
2175	7	"	9	122	
2175	7	"	8	110	
2175	7	"	9	101	
2175	7	"	3	44	
2406	22	good	10	104	moderately low Ca, low fat-soluble A.
2441	22	"	5	98	
2441	22	"	8	98	
2441	22	"	4.5	---	
2441	22	"	7	109	low fat-soluble A
2286	20	"	7.5	104	
2286	20	"	6	86	
2286	20	"	7	100	low water-sol. B
2286	20	"	9	104	
2286	20	"	4	60	
2405	22	"	7.8	109	low fat-soluble A
2405	22	"	8.0	81	
2405	22	"	2.5	60	

below 8,000,000 being 4,800,000. In the one lot (2750) on a maintenance diet in which both the calcium and fat-soluble A content of the diet was low, the erythrocyte counts on 3 animals were 2,500,000, 1,400,000, and 3,200,000 respectively. Since calcium is an important constituent of the red cell, a diet very low in calcium might tend to cause anemia.

In the 123 hemoglobin determinations, the highest content was 132 per cent, and the lowest 22 per cent. 66 of the rats (53 per cent) had a content above 100 per cent, and 97 (79 per cent) were above 90 per cent hemoglobin. Almost all the animals on the 9 per cent protein diets had a content of 100 per cent or more. The exceptions were those on diets containing a legume just as in the case of the lower erythrocyte counts. But even these animals did not have a hemoglobin content lower than the normal given for the rats at the Wistar Institute. The rats on the maintenance diets, however, had a somewhat lower content, 9 out of 14 determinations were below 90 per cent, the average being 61 per cent.

It is difficult to account for the tendency toward anemia exhibited by the animals on diets containing a legume. The legumes are as rich in iron as the cereals, and in some of those diets the legumes were supplemented by steak. It may be that the proteins of these diets lack one or more amino-acid necessary for the building up of the hemoglobin molecule. In the experimental work on the relation of iron and anemia this fact does not appear to be taken into account and it may explain in part at least the contradictory results obtained

by different investigators on the value of different forms of iron in the diet. (22).

THE NON-PROTEIN N OF THE BLOOD

Since Folin perfected his methods for the determination of non-protein N in blood, the literature has contained many references showing the importance of this factor. It is a means of detecting renal insufficiency in the removal of nitrogenous waste except in the case of unusually rapid absorption or of an abnormally high level of protein distinction such as occurs in fever. The amount of non-protein N in the blood of normal individuals is given by various writers as between 20 and 45 mgs. per 100 cc. It may be as high as 350 mgs. in 100 cc. blood in severe cases of uremia (23). Folin and Denis (1) state that in "strictly normal" material the range is only between 22 and 26 mgs. However, a "strictly normal" individual is a rarity. Persons without a specific kidney lesion gave readings between 24 mgs. and 62 mgs. per 100 cc. blood, with only two specimens below 26 mgs. They (1) also found that as individuals grew older the non-protein N of the blood increases.

For the normal rat Folin and Denis (24) give an average of 36 mgs. of non-protein N per 100 cc of blood. The following figures were obtained from normal male animals about 1 year old in this laboratory:

Table V.

	31.4 mgs.	per 100 cc. blood
	35.1 mgs.	ditto
	33.5 mgs.	"
	42.5 mgs.	"
	38.2 mgs.	"
	43.6 mgs.	"
	36.1 mgs.	"
Average	37 mgs.	"

Table VI.

Lot No.	Percentage protein	Character of protein	Non-protein N Mgs. per 100 cc (average of duplicates
2195	9	good	46.4
2195	9	"	49.8
2195	9	"	58.5
2191	9	poor	30.3
2191	9	"	44.0
2190	9	"	49.1
2190	9	"	56.4
2190	9	"	49.2
2190	9	"	50.2
2190	9	"	61.4
2190	9	"	39.3
2184	9	"	45.1
2184	9	"	36.7
2188	9	good	45.7
2188	9	"	53.5
2188	9	"	95.5
2193	9	poor	46.4
2193	9	"	49.8
2193	9	"	58.4
2180	9	"	52.7
2180	9	"	46.7
2180	9	"	50.0
2179	9	good	51.8
2179	9	"	61.9
2179	9	"	47.8
2177	9	poor	54.1
2177	9	"	53.3
2178	9	"	56.9
2178	9	"	48.1
2379	9	"	51.8
2378	9	good	41.4
2378	9	"	43.7
2371	9	poor	46.5
2371	9	"	60.4
2364	9	"	46
2364	9	"	52
2364	9	"	54.9
2369	9	"	39
2369	9	"	50.9
2363	9	"	41.3

Table VI. (continued).

Lot No.	Percentage protein	Character of protein	Non-protein N mgs. per 100 cc (average of duplicates.
2363	9	poor	60.7
2363	9	"	79.4
2346	9	good	61.7
2362	9	excellent	49
2362	9	excellent	42.8
2370	9	"	47.3
2370	9	"	42.4
2370	9	"	79.2
2370	9	"	57.3
2390	9	poor	47.7
2390	9	"	41.4
2390	9	"	49.2
2388	9	"	49
2388	9	"	42.8
2387	9	good	73.9
2387	9	"	66.5
2387	9	"	86.5
2389	9	poor	79.4
2181	9	good	56
2185	9	poor	40.9
2491	9	"	35.7
2491	9	"	67.5
2491	9	"	45.1
2491	9	"	49.7
2491	9	"	48.0
2494	9	"	67.8
2474	9	"	41.4
1894	8	"	92
1895	9	"	38.8
1896	10	"	71.8
1896	10	"	78.8
2437	16	"	48.9
2437	16	"	38.2
2437	16	"	85.6
2437	16	"	50.9
2437	16	"	62.5
2340	12	"	56.8
2340	12	"	43.7
2340	12	"	60.9
2343	18	good	48.6

Table VI. (continued).

Lot No.	Percentage protein	Character of protein	Non-protein N mgs. per 100 cc (average of duplicates).	
2343	18	good	45.2	
2343	18	"	45.1	
2414	35	"	46.6	
2417	20	fair	75.7	
2750	22	good	72.1	low fat-soluble A &
2750	22	"	75.9	low Ca.
2127	7 1/2	poor	37.1	
2127	7 1/2	"	41.8	
2127	7 1/2	"	39.8	
2127	7 1/2	"	54.1	
2175	7	"	57.4	
2175	7	"	49.7	
2406	22	good	45.7	mod. low Ca, low fat-
2406	22	"	38.3	sol. A.
2441	22	"	30.5	
2441	22	"	44.3	low A
2441	22	"	44.0	
2286	20	"	45.6	
2286	20	"	38.3	
2286	20	"	65.	
2286	20	"	55.3	
2405	22	"	52.7	
2405	22	"	62.1	low A
2405	22	"	63	

Duplicate determinations of non-protein N were made on 103 of the animals studied. The amount found varied from 30.5 mgs. to 92.0 mgs. per 100 cc. with an average of 50.6 mgs. Taking 45 mgs. as the upper level for the normal animal, 81 animals (79 per cent) had a non-protein N greater than this amount.

Since the diets of all these animals were conducive to premature senility, although in varying degree, we would expect to find a high level of non-protein N in the blood from the work of Folin (1) quoted above. No one diet appears to have an advantage over any other. Those in which the 9 per cent protein content was of the best quality, and those with a much higher content have about the same degree of variation from the normal. In general there is a fair agreement in the results obtained from animals on the same diet. There are a few exceptions, as for example in lots 2363 and 2070 in which the variations are from 41.3 mgs. to 79.4 mgs., and 42.4 mgs. to 79.2 mgs. respectively. However, a certain amount of variation is to be expected, especially among subnormal individuals. It is interesting to note that of the four animals on the Kaffir corn maintenance diet the three which are in poor condition have a high content, the fourth is in good condition and has a low content. Since the second and third generations raised on these diets are frequently very poor, it was thought that there might be a progressive increase in the non-protein N in these generations. This did not prove to be the case, as may be seen in Table VII.

Table VII.

First generation -	52.7 mgs. N	per 100 cc blood	(average of	74 animals)
Second "	51.6 mgs.	ditto	("	" 24 animals)
Third "	47.6 mgs.	"	("	" 4 animals)

There is no apparent relation between the number of days on the diets and the non-protein N of the blood. This is shown by Table VIII.

Table VIII.

200	days	on	diet	-	average	70.2 mgs.	non-protein	N	(2	animals)
250	"	"	"	-	"	47.5 mgs.	"	"	"	"
300	"	"	"	-	"	54.3 mgs.	"	"	"	"
350	"	"	"	-	"	45.6 mgs.	"	"	"	"
400	"	"	"	-	"	55.2 mgs.	"	"	"	"
500	"	"	"	-	"	48.1 mgs.	"	"	"	"
550	"	"	"	-	"	50.7 mgs.	"	"	"	"
600	"	"	"	-	"	55.4 mgs.	"	"	"	"
700	"	"	"	-	"	50.0 mgs.	"	"	"	"

Part IV.

SUMMARY.

1. Rats kept on a diet containing 9 per cent protein vary in some of their physiological reactions according to the quality of the protein. This is shown by differences in the rate of growth, in the time at which old age is apparent, the degree of fertility, the condition of the genital organs, the infant mortality rate, and to a lesser degree in the condition of the blood as shown by the erythrocyte count and the percentage of hemoglobin. However, the animals showed certain changes irrespective of the type of protein, demonstrating that the amount as well as the quality is significant. Almost all were in poor condition, the calcium metabolism was abnormal, and the average of the non-protein nitrogen of the blood was high. The lung infection which was so common appeared to occur independently of the type of diet.

2. A diet deficient in either fat-soluble A alone or in both calcium and fat-soluble A is associated with an abnormal calcium metabolism.

3. Animals on high levels of protein intake (up to 22 per cent) but on diets inadequate in other respects, may have a high non-protein nitrogen content in the blood, especially when they are in poor condition.

Part V.

CONCLUSIONS.

A diet containing 9 per cent protein will not permit the rat to complete a normal life cycle. The degree to which this is accomplished depends on the quality of the protein mixture.

A deficiency in fat-soluble A unfavorably influences the calcium metabolism. Factors in the diet other than the protein content also influence the amount of non-protein nitrogen in the blood.

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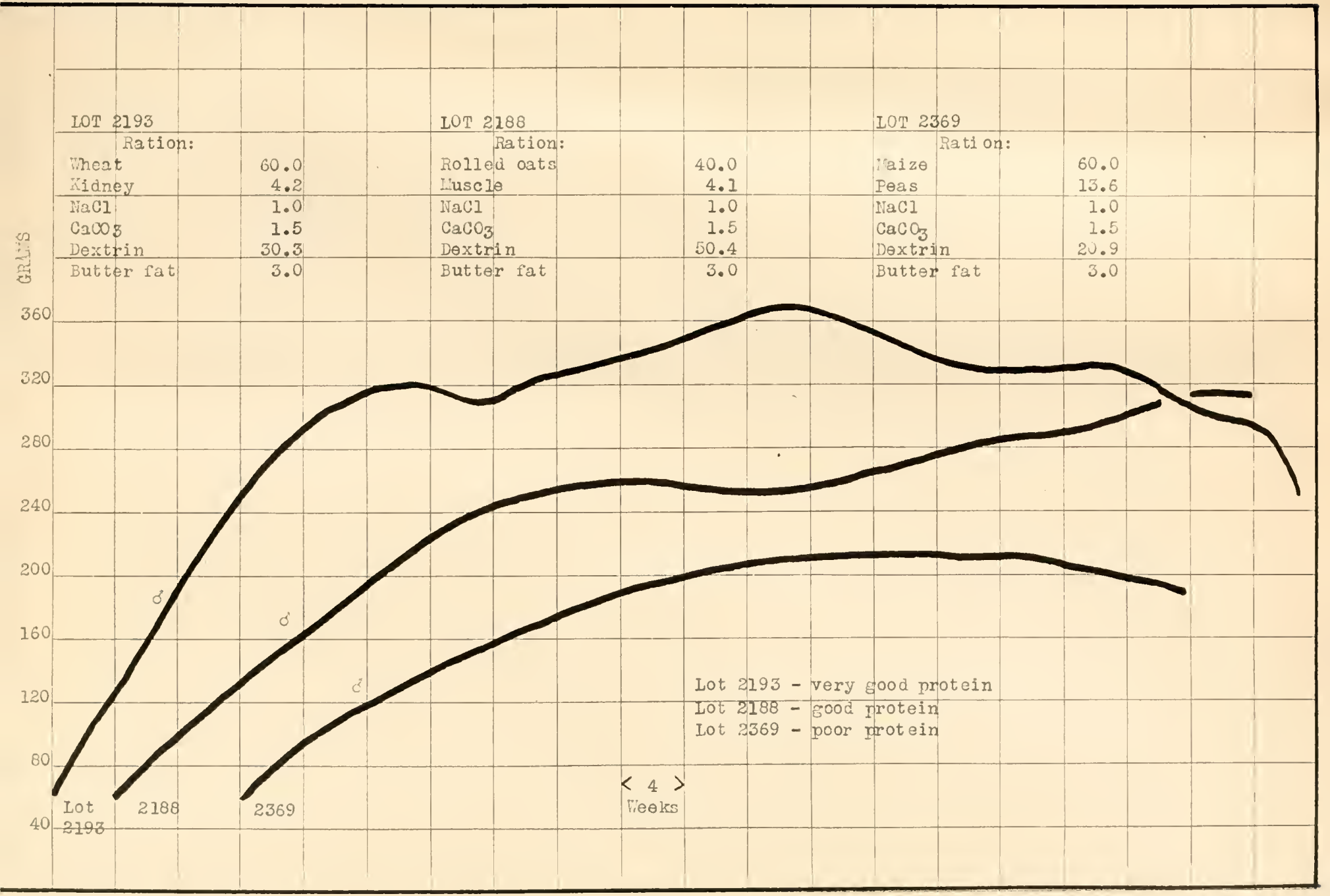
BIOGRAPHY

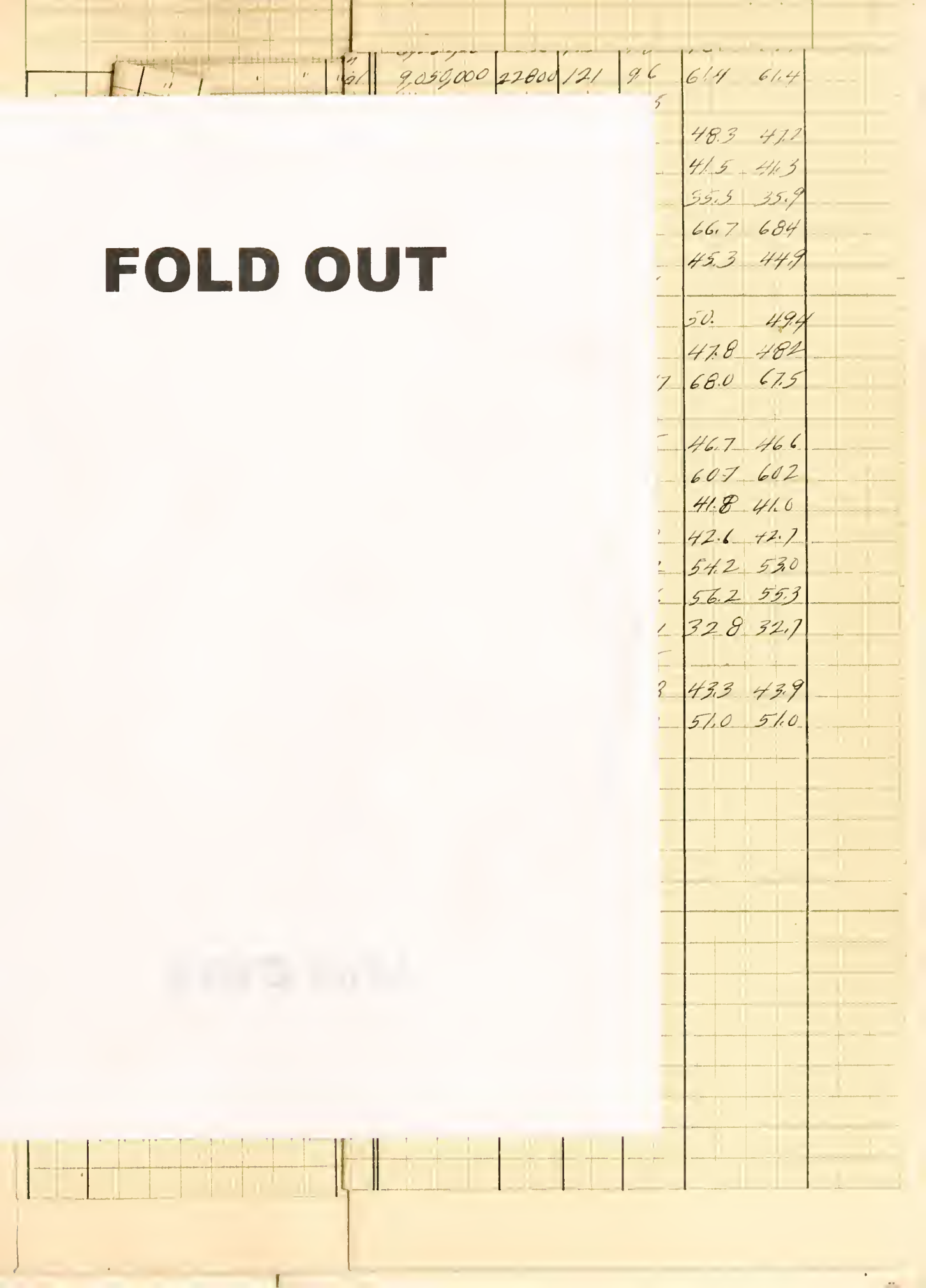
The writer was born in Philadelphia, Pa., on December 11, 1894. She attended the public schools of Philadelphia and Reading, Pa., and was graduated from the Girls' High School of Reading in 1911. In 1915 she received the degree of Bachelor of Science in Chemistry from the Pennsylvania State College.

From 1915 to 1917 she was employed as a chemist at the Baltimore City Health Department, and during 1916 and 1917 was also a graduate student at the Medical School, Johns Hopkins University. In the latter part of 1917 she was specialist in food research in the United States Department of Agriculture, but was transferred to the United States Food Administration in January, 1918, to serve on the editorial board of the collegiate section. She was instructor and fellow during 1918-1919 and 1919-1920 respectively in the Department of Chemical Hygiene, School of Hygiene and Public Health, Johns Hopkins University, and a graduate student at the University of Chicago during the summer quarter of 1919.

She was granted leave of absence in 1920 to serve as specialist in nutrition in the Extension Service of the New Jersey State College.

LOT 2193		LOT 2188		LOT 2369	
Ration:		Ration:		Ration:	
Wheat	60.0	Rolled oats	40.0	Maize	60.0
Kidney	4.2	Muscle	4.1	Peas	13.6
NaCl	1.0	NaCl	1.0	NaCl	1.0
$CaCO_3$	1.5	$CaCO_3$	1.5	$CaCO_3$	1.5
Dextrin	30.3	Dextrin	50.4	Dextrin	20.9
Butter fat	3.0	Butter fat	3.0	Butter fat	3.0

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42.7
54.2
53.0
56.2
55.3
32.8
32.7
43.3
43.9
51.0
51.0

	Diet *	Blood findings				
		Erythrocytes	Leucocytes	Hb	Vol Mgl Per 100cc Blood	Non Protein Mgs per 100cc Blood
						23.0 90.7
						22 38.8
						71.4 72.2
					17.8	78.4 78.2
					8.1	44.9 40.1
					9	78.5 78.3
						65 65
					9	35.5 35.7
					18.5	57.7 56.0
						44.1 43.5
						61.4 60.2
						48.9 48.2
					8.2	45.2 45.7
					12.3	45.1 45.1
					18.6	52.4 52.6
						62.2 62.0
					14	63 63
					6.2	45.7 45.7
					12.2	38.5 38.1
					7.1	47.0 46.2
					7.6	75.7 75.7
						49.1 48.8
					10.9	38.3 38.1
						86. 85.2
						50.9 50.2

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